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Antifungal Susceptibility Testing of *Malassezia* spp. with an Optimized Colorimetric Broth Microdilution Method

Cheryl Leong, Antonino Buttafuoco, Martin Glatz, Philipp P. Bosshard

Department of Dermatology, University and University Hospital of Zurich, Zurich, Switzerland

ABSTRACT *Malassezia* is a genus of lipid-dependent yeasts. It is associated with common skin diseases such as pityriasis versicolor and atopic dermatitis and can cause systemic infections in immunocompromised individuals. Owing to the slow growth and lipid requirements of these fastidious yeasts, convenient and reliable antifungal drug susceptibility testing assays for *Malassezia* spp. are not widely available. Therefore, we optimized a broth microdilution assay for the testing of *Malassezia* that is based on the CLSI and EUCAST assays for *Candida* and other yeasts. The addition of ingredients such as lipids and esculin provided a broth medium formulation that enabled the growth of all *Malassezia* spp. and could be read, with the colorimetric indicator resazurin, by visual and fluorescence readings. We tested the susceptibility of 52 strains of 13 *Malassezia* species to 11 commonly used antifungals. MIC values determined by visual readings were in good agreement with MIC values determined by fluorescence readings. The lowest MICs were found for the azoles itraconazole, posaconazole, and voriconazole, with MIC_{90} values of 0.03 to 1.0 μg/ml, 0.06 to 0.5 μg/ml, and 0.03 to 2.0 μg/ml, respectively. All *Malassezia* spp. were resistant to echinocandins and griseofulvin. Some *Malassezia* spp. also showed high MIC values for ketoconazole, which is the most widely recommended topical antifungal to treat *Malassezia* skin infections. In summary, our assay enables the fast and reliable susceptibility testing of *Malassezia* spp. with a large panel of different antifungals.

KEYWORDS antifungal agents, antifungal susceptibility testing, fungi, *Malassezia*, yeasts

Malassezia is a genus of lipid-dependent yeasts that currently includes 14 species. With the exception of *Malassezia pachydermatis*, *Malassezia* species are lipid dependent. *Malassezia sympodialis*, *Malassezia slooffiae*, *Malassezia furfur*, *Malassezia globosa*, *Malassezia restricta*, *Malassezia obtusa*, *Malassezia japonica*, *Malassezia dermatis*, and *Malassezia yamatoensis* are considered anthropophilic, as they have been isolated from human skin (1). *Malassezia* is the most common fungal genus of the healthy human skin microbiome (2). Additionally, a pathogenic role is attributed to these yeasts in common skin diseases such as pityriasis versicolor, atopic dermatitis, and seborrheic dermatitis, and they can cause severe systemic infections in neonates and immunocompromised individuals (3, 4).

In infectious diseases, antimicrobial susceptibility testing is a useful tool to determine the appropriate antimicrobial treatment, particularly if antimicrobial susceptibility cannot be predicted based on the identity of the infectious agent (5). Treatment of *Malassezia*-related infections has thus far relied on the predicted susceptibility to common antifungals such as topical azoles. However, the antifungal treatment of *Malassezia*-associated skin diseases is not clinically efficacious in up to one-third of patients (6). This may be attributable to malcompliance as well as the antifungal resistance of *Malassezia* spp. Moreover, knowledge regarding the susceptibility of

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Address correspondence to Martin Glatz, martin.glatz@usz.ch, or Philipp P. Bosshard, philipp.bosshard@usz.ch.

M.G. and P.P.B. contributed equally to this work.

different *Malassezia* species and strains to particular antifungals is scarce (7–10), which supports the need for antifungal susceptibility testing for *Malassezia* spp. Existing assays for determination of the MIC values for antifungals, as recommended by the CLSI (11) and EUCAST (http://www.eucast.org) guidelines, are applicable only for fast-growing fungi such as *Candida* spp. These assays are not suitable for *Malassezia* spp., which are slow growing and fastidious. Therefore, some studies that tested the antifungal susceptibility of *Malassezia* spp. used assays modified from the CLSI and EUCAST guidelines. The modifications included variations in growth medium composition and inoculum size (Table 1) (7–9). Turbidity was the preferred readout method for most of the assays (7, 8). However, drawbacks of turbidity readings include limited reproducibility and the possibility of underestimating MIC values (12). To overcome these limitations, we have developed a new antifungal susceptibility assay, based on a broth medium, that allows growth and reliable and convenient antifungal testing of all *Malassezia* species. It is compatible with the fluorometric indicator resazurin, enabling the rapid and effective determination of MIC values by visual and fluorescence readings.

RESULTS

Growth of *Malassezia* spp. in OptiMAL broth medium. Serial dilutions of each broth medium component were tested to determine the optimal concentrations for five *Malassezia* species, i.e., *M. sympodialis*, *M. slooffiae*, *M. furfur*, *M. globosa*, and *M. pachydermatis*. Optimal glycerol and Tween 60 concentrations were 0.25 to 0.5% and 0.5%, respectively. We used a concentration of 0.05 to 2% oleic acid for OptiMAL, because most *Malassezia* species preferred oleic acid to olive oil in assimilation assays. Glucose concentrations of more than 2% did not improve growth. The optimal pH range was pH 6.0 to 6.5.

In the absence of sodium bicarbonate, RPMI and RPMI++ tended to color bleach. Testing growth with sodium bicarbonate concentrations ranging from 0 to 32 mg/ml showed that *Malassezia* spp. were able to tolerate sodium bicarbonate concentrations of up to 2 mg/ml before growth inhibition occurred. A sodium bicarbonate concentration of 2 mg/ml was optimal for visual and fluorescence readings, because it boosted color intensity and improved pH buffering (see Fig. S1 in the supplemental material). The addition of 0.1 mg/ml esculin increased the fluorescence signal by 2- to 4-fold (Fig. S1). These observations support the use of sodium bicarbonate and esculin as useful broth medium additives that are compatible with resazurin for *Malassezia* growth and broth microdilution assays.

Optimal inoculum size for *Malassezia* spp. By testing a range of inoculum sizes and varying the incubation time before the readings, we observed that the MIC values of some antifungals depended strongly on the inoculum size and the incubation time, while other antifungals were less affected. For amphotericin B, growth curves were not influenced by the inoculum size or the incubation time (Fig. S2, left column). However, growth curves were less reproducible with other antifungals, especially when an inoculum of >100,000 CFU/ml was used. To provide reproducible results for all antifungals, we decided to use a final inoculum of 5.0×10^3 to 5.0×10^4 CFU/ml for all tested *Malassezia* strains.

Incubation times of 18 to 48 h gave comparable growth curves, and MIC values determined at those time points did not differ by more than 1 to 2 dilution steps for inocula of 5.0×10^3 to 5.0×10^4 CFU/ml (Fig. S2, right column). Color development after 6 to 12 h of incubation was insufficient for reliable MIC readings, while the fluorescence signal faded after 60 h of incubation. Therefore, we decided to read the plates after 24 to 48 h of incubation. Ketoconazole was the only exception, for which incubation periods of 24 to 36 h resulted in a loss of growth curve sensitivity and high MIC readings.

Agreement between visual and fluorescence readings. We were interested in assessing whether MIC values determined by visual readings differed from MIC values determined by fluorescence readings. MIC values determined by fluorescence readings

TABLE 1 Synopsis of studies investigating broth microdilution assays for the antifungal susceptibility testing of *Malassezia* spp.

Reference	Species	Antifungals	Broth medium composition	Inoculum size (CFU/ml)	Incubation time (days)	Incubation temperature (°C)	Reading method
Our study	52 strains; *M. sympodialis*, *M. slooffiae*, *M. furfur*, *M. globosa*, *M. restricta*, *M. obtusa*, *M. dermatis*, *M. japonica*, *M. yamatoensis*, *M. pachydermatis*, *Malassezia nana*, *M. caprae*, *Malassezia cuniculi*	Amphotericin B, terbinafine, ketoconazole, fluconazole, posaconazole, itraconazole, voriconazole, caspofungin, micafungin, anidulafungin, griseofulvin	RPMI 1640 medium with 0.165 M MOPS, 0.2% sodium bicarbonate, 0.5% glycerol, 0.5% Tween 60, 2% oleic acid, and 1% esculin	5.0×10^3 to 5.0×10^4	2 or 3	35	Visual (color) and fluorescence
Rojas et al. (7)	*M. furfur* (39 strains), *M. sympodialis* (20 strains), *M. globosa* (14 strains)	Fluconazole, ketoconazole, voriconazole, itraconazole, amphotericin B, miconazole	RPMI 1640 medium with 1.8% glucose, 1% peptone, 0.5% ox bile, 0.5% malt extract, 1% glycerol, 0.5% Tween 40, 0.05% Tween 80	0.5×10^5 to 2.5×10^5	3 or 4	32	Turbidity
Velegraki et al. (8)	53 strains; *M. furfur*, *M. pachydermatis*, *M. sympodialis*, *M. slooffiae*, *M. globosa*, *M. restricta*, *M. dermatis*	Amphotericin B, itraconazole, fluconazole, voriconazole, ketoconazole, terbinafine, posaconazole	RPMI 1640 medium with 20 g glucose, 4 g ox bile, 1 ml glycerol, 0.5 g glycerol monostearate, and 0.4 ml Tween 20	2.0×10^3 to 3.5×10^3 or 3.0×10^3 to 4.0×10^3	*M. furfur*, 2; *M. pachydermatis*, 2; *M. sympodialis*, 3; *M. slooffiae*, 3; *M. globosa*, 3; *M. restricta*, 3; *M. dermatis*, 3	32	Turbidity
Miranda et al. (9)	*M. furfur* (74 strains), *M. sympodialis* (11 strains), *M. obtusa* (8 strains), *M. globosa* (2 strains)	Fluconazole, ketoconazole, itraconazole, voriconazole	Modified Leeming-Notman medium containing 0.1% glucose, 0.1% peptone, 0.8% bile salts, 0.2% yeast extract, 0.1%glycerol, 0.5% Tween 60, and 3% olive oil	$2.5 \times 10^3 \pm 1.0 \times 10^3$	*M. furfur*, 3; *M. sympodialis*, 3; *M. globosa*, 5; *M. obtusa*, 5	32	Turbidity
Gupta et al. (10)[a]	55 strains; *M. furfur*, *M. sympodialis*, *M. slooffiae*, *M. pachydermatis*, *M. globosa*, *M. obtusa*, *M. restricta*	Ketoconazole, voriconazole, itraconazole, terbinafine	Diagnostic Sensitivity Testing agar and glycerol	1.0×10^4	7	35	Colony growth on agar plates

[a]This assay was performed using agar-based medium.

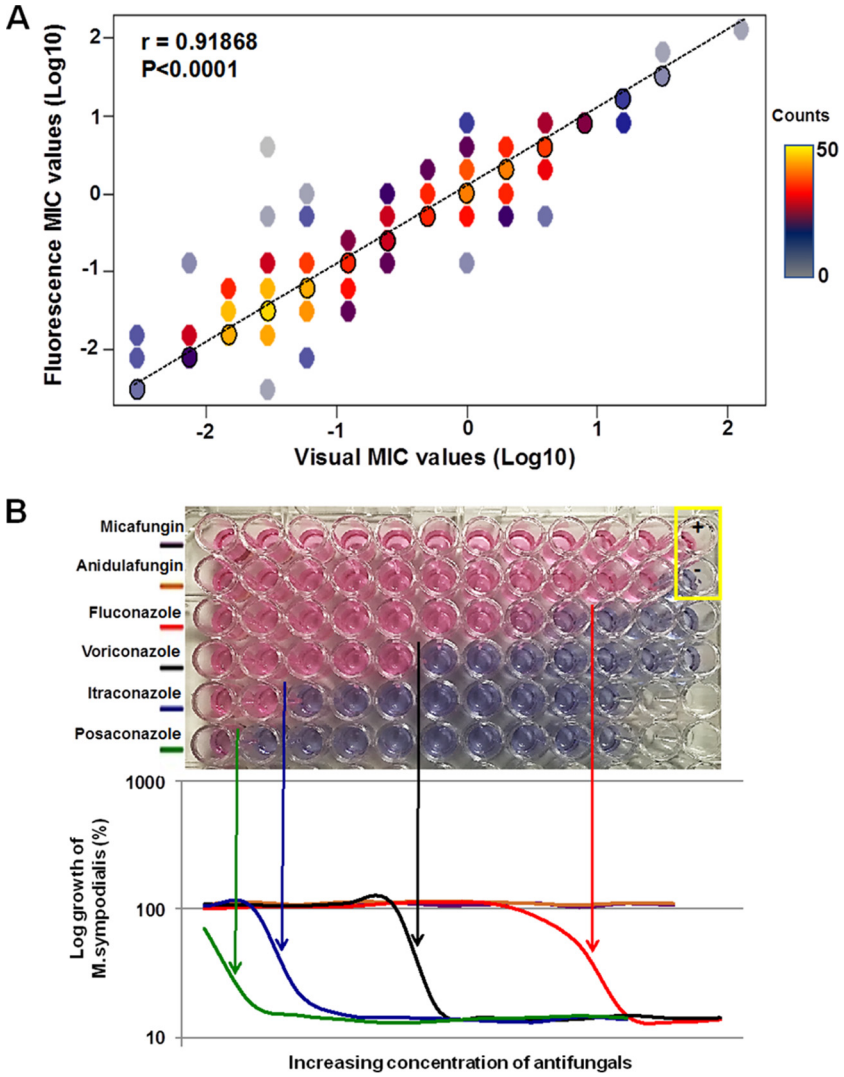

FIG 1 (A) MIC values of antifungals (except griseofulvin and echinocandins) for 52 *Malassezia* strains, determined by visual readings (*x* axis) and fluorescence readings (*y* axis). Black circles represent points at which the two data readings were in 100% agreement. The number of MIC values per data point in the graph is indicated by the color bar. Agreement between visual and fluorescence readings was determined by Pearson correlation analysis. (B) Agreement between MIC values determined by visual readings and fluorescence readings from a representative antifungal resistance assay plate testing *M. sympodialis*. Each row represents a particular antifungal, with concentrations increasing from left to right. In the yellow box, the plus sign indicates the positive control without antifungal and the minus sign indicates the negative control containing broth medium without *M. sympodialis*. Pink wells indicate the growth of *M. sympodialis*, and blue wells indicate no growth. The graph represents the fluorescence readings of the color changes.

appeared slightly higher than those determined by visual readings, but the difference was not significant ($P = 0.25$). Overall, the MIC values determined by the two readout methods were strongly correlated with each other for all antifungals (Pearson's correlation coefficients of >0.857 for all drugs) (Fig. 1). For most antifungals, MIC values determined by the two methods were within 2 dilution steps in 96 to 100% of cases (Table 2). The agreement of MIC values for ketoconazole was slightly lower, with MIC values within 2 dilution steps in 88% of cases (Table 2).

Susceptibility of *Malassezia* spp. to antifungals. We assessed the antifungal susceptibility profiles of 52 *Malassezia* strains, including 13 reference strains and 39 clinical strains (Table 3), with 11 antifungals commonly used in clinical practice. The lowest MIC values were found for itraconazole, posaconazole, and voriconazole for most strains tested (Fig. 2 and Table 4). Among the azoles, the highest MIC values and

TABLE 2 Agreement of MIC values from visual and fluorescence readings

Agreement level	Agreement (%)						
	Amphotericin B	Terbinafine	Ketoconazole	Fluconazole	Itraconazole	Posaconazole	Voriconazole
MICs identical	75	73	63	67	67	73	75
MICs within 1 dilution	96	94	84	88	88	94	96
MICs within 2 dilutions	98	96	88	96	98	100	98

the widest MIC ranges were observed for fluconazole (Table 4; also see Table S1). Ketoconazole showed low MICs for most strains but very high MICs for a few strains (Fig. 2 and Table 4). MICs for terbinafine and amphotericin B showed wide ranges; some strains showed low MICs while others had very high MIC values. All strains had very high MIC values for the echinocandins anidulafungin, caspofungin, and micafungin, as well as griseofulvin, suggesting an intrinsic resistance of *Malassezia* spp. to these antifungals (Fig. 2); therefore, MIC values for these antifungals are not shown in Tables 2 and 4. Two species, namely, the anthropophilic *M. obtusa* and the zoophilic *Malassezia caprae*, appeared to be resistant to most antifungals (Fig. 2 and Table 4). Terbinafine was the only antifungal that was effective against *M. caprae*.

DISCUSSION

RPMI 1640 medium containing 0.165 M 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer is the basis for the broth microdilution methods recommended by the CLSI and EUCAST (6, 7). This broth is not optimal for the growth of fastidious fungi such as *Malassezia* spp., which require lipid supplementation. By adding multiple components, including lipids (such as oils and Tween compounds) and esculin, to our broth medium, we facilitated the growth of *Malassezia* spp. We have named this new broth medium OptiMAL. OptiMAL may also be suitable for other yeast species, such as *Candida*, that utilize additives such as Tween compounds (13).

Resazurin (also known as alamarBlue) is used as an indicator in the colorimetric YeastOne Sensititre assay (Thermo Scientific, Zug, Switzerland) for routine antifungal susceptibility testing of yeasts and molds such as *Candida* spp. and *Aspergillus* spp. (14, 15). It changes from blue to pink as a result of growth (16) and allows for the visual and spectrophotometric determination of MIC values. Until now, it has not been used in broth media for *Malassezia* spp. because it is not always color stable during prolonged incubation and therefore needs to be buffered (16, 17). The careful buffering of OptiMAL with sodium bicarbonate and esculin allowed for the concurrent use of resazurin as a color indicator. The use of esculin in OptiMAL improved the growth of all *Malassezia* species by up to 4-fold (Fig. S1), and it was a key ingredient to improve the

TABLE 3 *Malassezia* species and proposed inocula for antifungal susceptibility testing

Strain no.	Species	Host	No. of strains tested	Reference strain	OD_{600} for inoculum[a]
1	*M. sympodialis*	Human	24	CBS 7222	0.1
2	*M. slooffiae*	Human	10	CBS 7956	0.05
3	*M. furfur*	Human	5	CBS 1878	0.005
4	*M. globosa*	Human	3	CBS 7966	0.2–1
5	*M. restricta*	Human	2	CBS 7877	0.2–1
6	*M. obtusa*	Human	2	CBS 7876	0.2–1
7	*M. dermatis*	Human	1	CBS 9169	0.1
8	*M. japonica*	Human	1	CBS 9432	0.2
9	*M. yamatoensis*	Human	1	CBS 9725	0.02
10	*M. nana*	Animal	1	CBS 9557	0.1
11	*M. pachydermatis*	Animal	1	CBS 1879	0.01
12	*M. cuniculi*	Animal	1	CBS 11721	0.1
13	*M. caprae*	Animal	1	CBS 10434	0.1

[a]OD_{600} values for broth microdilution testing, as determined by plating 10 μl of a 1:10 dilution to achieve inocula of 10^4 to 10^5 CFU/ml. For the susceptibility assay, 50 μl of inoculum was added to 50 μl of 2× concentrations of antifungals, to achieve final cell densities of 5.0×10^3 to 5.0×10^4 CFU/ml.

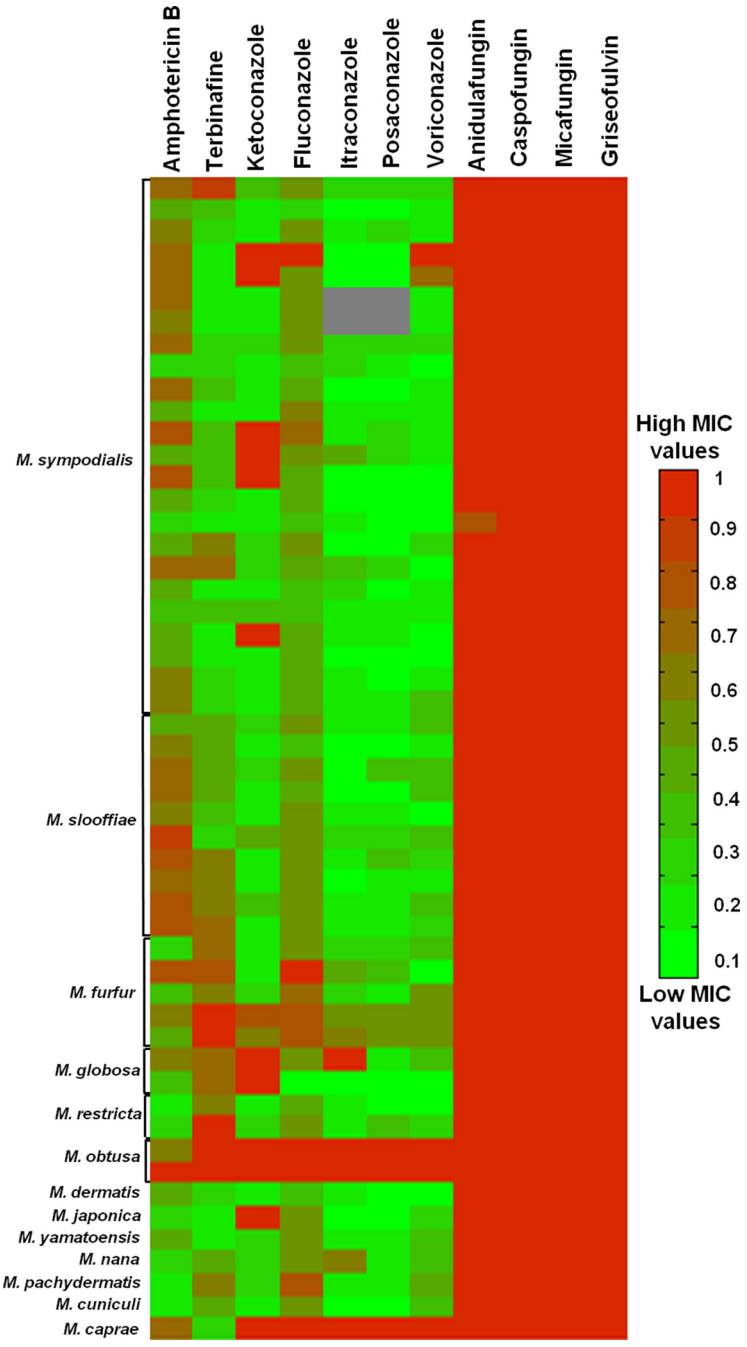

FIG 2 MIC values of 11 antifungals for 52 *Malassezia* strains, as determined by visual readings. MIC values were normalized from 0 to 1, with 1 being the highest antifungal concentration tested. The first row for each *Malassezia* species represents the susceptibility profile of the reference strain. Gray sections indicate inconclusive MIC results.

accuracy and reproducibility of our assay. Studies on fungal growth usually omit sodium bicarbonate because it causes growth inhibition of many fungal species (18). We observed that *Malassezia* spp. grew well in OptiMAL containing 2 mg/ml sodium bicarbonate. The bicarbonate served as a complementary buffer to MOPS; it boosted the intensity of the resazurin color change and yielded higher sensitivity of fluorescence detection.

The lack of standardized guidelines for drug susceptibility testing in *Malassezia* spp. has led some studies (8, 9) to use small inocula, in accordance with the guidelines

TABLE 4 MIC ranges and mean MICs of antifungals determined by visual readings

Species	MIC range (mean MIC) (μg/ml)						
	Amphotericin B	Terbinafine	Ketoconazole	Fluconazole	Itraconazole	Posaconazole	Voriconazole
M. sympodialis (24 strains)	0.125–4 (1.27)	≤0.125 to 16 (1.15)	≤0.03 to >4 (1.03)	0.25 to >128 (7.09)	≤0.015 to >8 (0.37)	≤0.015 to >8 (0.36)	≤0.003 to >8 (0.38)
M. slooffiae (10 strains)	0.5 to >8 (2.85)	0.25–4 (1.48)	≤0.03 to 0.125 (0.07)	0.5–4 (1.95)	≤0.015 to 0.06 (0.04)	≤0.015 to 0.125 (0.03)	≤0.003 to 0.03 (0.02)
M. furfur (5 strains)	0.125–4 (1.18)	2 to >16 (9.2)	≤0.03 to 2 (0.52)	4 to >128 (42.4)	0.06–1 (0.37)	0.03–0.5 (0.24)	0.03–2 (0.51)
M. globosa (3 strains)	0.25–1	4	>4	4	≤0.015 to >8	≤0.015 to 0.03	≤0.003 to 0.03
M. restricta (2 strains)	≤0.06 to >0.125	2 to >16	≤0.03 to 0.06	1–4	0.03	≤0.015 to 0.125	≤0.003 to 0.015
M. obtusa (2 strains)	1 to >8	>16	>4	>128	>8	>8	>8
M. dermatis (1 strain)[a]	0.5	0.25	≤0.03	0.5	0.03	≤0.015	≤0.003
M. japonica (1 strain)[a]	0.125	≤0.125	>4	2	≤0.015	≤0.015	0.015
M. yamatoensis (1 strain)[a]	0.5	≤0.125	0.06	4	0.06	0.03	0.03
M. pachydermatis (1 strain)[a]	≤0.06	2	0.06	32	0.03	0.03	0.06
M. caprae (1 strain)[a]	2	0.25	>4	>128	>8	>8	>8
M. nana (1 strain)[a]	0.125	1	0.06	4	0.06	1	0.03
M. cuniculi (1 strain)[a]	≤0.06	1	≤0.03	4	≤0.015	≤0.015	0.03

[a]No range; only the reference strain was tested for these species.

specified by the CLSI for yeasts (11), because overtly large inocula are associated with inaccurate MIC values (19). However, the EUCAST guidelines for yeasts use a 100-fold larger inoculum than do the CLSI guidelines. To test the relevance of these differences between the CLSI and EUCAST guidelines for *Malassezia* spp., we performed inoculum titration studies. We decided on 5.0×10^3 to 5.0×10^4 CFU/ml as the optimal inoculum size for *Malassezia* broth microdilution assays, because this size gave reproducible MIC values within 2 dilution steps for repeats with the same *Malassezia* strain. Ketoconazole was particularly sensitive to variations in inoculum size and incubation time.

Visual readings for changes in color and turbidity are the most common form of MIC determinations. Quantitative readings can be performed by measuring absorbance or fluorescence, with the latter being more sensitive (20). In general, visual determinations of color changes may be preferable to absorbance or fluorescence readings in laboratory practice, because they do not require additional technical equipment, are faster, and therefore are cheaper (12). Few studies have investigated the agreement of MIC values determined by visual and fluorescence readings. For example, a comparison of MIC values of azoles for 88 *Aspergillus fumigatus* isolates determined by absorbance and visual turbidity reading were in good agreement (21). Neither absorbance nor turbidity readings were an option in our assay, however, because (i) some very slow-growing species such as *M. furfur* would have required much larger inocula than recommended to visualize turbidity; (ii) the clumpiness of some species, such as *M. globosa* and *M. restricta*, hampered reliable accurate absorbance or turbidity readings; and (iii) our assay is based on resazurin, and turbidity readings are not recommended when resazurin is used (22). Therefore, we decided to use visual color changes and fluorescence readings to determine MIC values. Overall, our MIC values determined by visual and fluorescence readings were in good agreement. Slightly higher MIC values were obtained with fluorescence readings, presumably because of the higher sensitivity of fluorescence readings. These differences were generally within 1 to 2 dilution steps, which is acceptable by CLSI and EUCAST standards. Our findings substantiate the concordance of visual and fluorescence readings for MIC values in resazurin-based assays.

Despite the use of an optimized growth medium and a standardized inoculum size, repeated testing (at least duplicates) showed that the azoles, and to a lesser extent terbinafine, might be prone to considerable test-to-test variations. In particular, ketoconazole testing required more repeats. This may be attributable to (i) the antagonization of medium components (10) and/or (ii) the susceptibility of ketoconazole to variations in inoculum size and incubation time, as described above.

We have applied this optimized broth microdilution method to test the susceptibility of 52 *Malassezia* strains to 11 antifungals. Our assay included the echinocandin class as well as griseofulvin. The data showed that *Malassezia* is resistant to these antifungals, which is in line with literature reports (3, 23). The azoles itraconazole, posaconazole, and voriconazole appeared to be the most effective antifungals against *Malassezia* spp. High MIC values for these azoles were rare, which is in line with previous studies (10, 24). Most of our *Malassezia* strains were susceptible to concentrations of fluconazole and ketoconazole described previously (7–9, 25). However, some strains were resistant to ketoconazole. This could be of clinical significance, because ketoconazole is the first-line topical antifungal in the treatment of *Malassezia*-associated skin diseases such as pityriasis versicolor or seborrheic dermatitis (26). Terbinafine, a widely used allylamine, is active against dermatophytes and molds and can be applied topically or systemically (27). It is not recommended as a first-line treatment for *Malassezia*-associated diseases (26). The relatively low MIC values of terbinafine against many of our *Malassezia* strains suggest that terbinafine may be a treatment alternative for *Malassezia* infections that do not respond to azoles.

In summary, we have developed an optimized broth microdilution assay, compatible with the colorimetric indicator resazurin, for the fast and efficient profiling of antifungal susceptibility in *Malassezia* spp. Determinations of MIC values by visual readings of color changes versus fluorescence readings were comparably reliable.

TABLE 5 Broth media for *Malassezia* spp.

Component	RPMI+[a]	RPMI ++	RPMI++ with esculin (OptiMAL)
MOPS (M)	0.165	0.165	0.165
Sodium bicarbonate (mg/ml)	0.2	0.2	0.2
Glucose (% of volume)	2	2	2
Glycerol (% of volume)	0.1	1	0.5
Glycerol monostearate (% of volume)	0.05		
Tween 20 (% of volume)	0.04		
Tween 60 (% of volume)		0.5	0.5
Olive oil			
Oleic acid (% of volume)		2	2
Esculin (% of volume)			0.1
Resazurin (μg/ml)	12.5	12.5	12.5
pH	6.32	6.25	6.26

[a]Adapted from Velegraki et al. (8).

Among the 52 *Malassezia* strains tested, azoles such as voriconazole, itraconazole, and posaconazole were the most effective antifungals. Terbinafine might be a treatment alternative for *Malassezia* infections.

MATERIALS AND METHODS

***Malassezia* strains.** *Malassezia* reference strains were obtained from the Centraalbureau voor Schimmelcultures (CBS) (Utrecht, The Netherlands) (Table 3). Clinical strains were obtained from patients who were seen at the Department of Dermatology, University Hospital of Zurich (Zurich, Switzerland). All strains were maintained on modified Leeming-Notman (mLN) agar and were identified as described previously (28, 29).

Optimization of broth medium formulation. The basis of the broth medium was filtered RPMI 1640 medium with 0.165 M MOPS without sodium bicarbonate (Sigma-Aldrich, Zurich, Switzerland). Sodium bicarbonate, glucose, Tween 60, oleic acid, glycerin, and esculin (Sigma-Aldrich) were selectively titrated and added to the broth medium. Reference strains of *M. sympodialis*, *M. slooffiae*, *M. furfur*, *M. globosa*, and *M. pachydermatis* were inoculated in triplicate at the stipulated optical density (OD) (Table 3) and incubated for 48 h at 35°C. Growth was assessed by scoring the turbidity of the broth medium on an arbitrary scale ranging from 0 (no growth) to 4 (highest growth). This led to the formulation of three candidate media (Table 5). RPMI++ with esculin provided the best conditions for growth and colorimetric readings for all tested *Malassezia* strains. We have named this medium OptiMAL and used it for antifungal susceptibility testing.

***Malassezia* inoculum quantification and optimization.** *Malassezia* inocula were quantified in suspension by measurements of the OD at 600 nm (OD_{600}) (Cytation 3 plate reader; BioTek, Winooski, VT, USA). To correlate OD_{600} readings with the numbers of yeast cells, serial dilutions of the suspensions were plated on mLN agar at 35°C and the CFU were counted after 4 to 7 days.

To determine the optimal inoculum range for *Malassezia* spp., five different inocula (approximately 2,500, 5,000, 50,000, 100,000, and 200,000 CFU/ml) of *M. furfur* CBS 1878 were tested together with seven antifungals (amphotericin B, terbinafine, ketoconazole, fluconazole, itraconazole, posaconazole, and voriconazole). Plates were read every 6 h for the first 24 h and subsequently at 12-h intervals for up to 60 h, as described below.

Broth microdilution assay. Antifungals tested in the assay were amphotericin B (concentration range, 0.06 to 8 μg/ml), caspofungin (0.06 to 8 μg/ml), griseofulvin (0.06 to 8 μg/ml), terbinafine (0.125 to 16 μg/ml), fluconazole (0.06 to 128 μg/ml), ketoconazole (0.03 to 4 μg/ml), itraconazole (0.015 to 8 μg/ml), posaconazole (0.015 to 8 μg/ml), voriconazole (0.003 to 8 μg/ml) (all from Sigma-Aldrich), micafungin, and anidulafungin (all from MedChemExpress Europe, Sollentuna, Sweden). Serial 2-fold dilutions of 200× stocks of antifungals were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at −80°C. Inocula (50 μl) were added to 50 μl of 2× concentrated antifungals to achieve a final cell density of 5.0×10^3 to 5.0×10^4 CFU/ml. For inoculum verification, 10 μl of a 1:10 diluted inoculum was plated onto mLN agar and incubated for 4 to 7 days at 35°C. MIC values from each well plate were deemed reliable only if counts on agar plates were within 10 to 100 CFU.

Candida parapsilosis ATCC 22019 was used as a quality control strain to assess the accuracy of our antifungal dilutions and the reproducibility of results for fluorescence and visual readings, according to CLSI (11) and EUCAST (http://www.eucast.org) guidelines.

Analysis of antifungal susceptibility assay data. Plates were analyzed when the indicator resazurin (Sigma-Aldrich) changed from blue to pink in the positive control. The color change was assessed by (i) visual reading and (ii) fluorescence analysis at 530 nm and 590 nm (Cytation 3 plate reader). Growth was measured relative to the positive control. MIC values for azoles and echinocandins were defined as 50% growth inhibition and those for amphotericin B as complete growth inhibition, in accordance with CLSI standards (11). For terbinafine, the MIC was defined as the lowest drug concentration that completely inhibited growth.

For each strain, at least duplicate testing was performed. If the MIC values differed by more than 2 dilution steps, then experiments were repeated. In cases in which repeat testing gave MIC values on extreme ends of the dilution scale, an inconclusive MIC result was recorded.

Heat maps of the final MIC values for each strain were plotted using the minimum and maximum drug ranges tested, normalized on a scale of 0 to 1. Analysis of variance (ANOVA) and correlation analysis were applied by calculating Pearson's *r* values. Inferential statistics were calculated with StatPlus:mac 2016 (AnalystSoft Inc., Walnut, CA, USA), and *P* values of <0.05 were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00338-17.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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We declare no conflicts of interest.

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